Cell Surface Proteoglycan Associates with the Cytoskeleton at the Basolateral Cell Surface of Mouse Mammary Epithelial Cells

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Abstract. The cell surface proteoglycan on normal murine mammary gland mouse mammary epithelial cells consists of an ectodomain bearing heparan and chondroitin sulfate chains and a lipophilic domain that is presumed to be intercalated into the plasma membrane. Because the ectodomain binds to matrix components produced by stromal cells with specificity and high affinity, we have proposed that the cell surface proteoglycan is a matrix receptor that binds epithelial cells to their underlying basement membrane. We now show that (*a*) the proteoglycan surrounds cells grown in subconfluent or newly confluent monolayers, but becomes restricted to the basolateral surface of cells that have been confluent for a week or more; (*b*) Triton X-100 extraction distinguishes three fractions of cell surface proteoglycan: a fraction released by detergent and presumed to be free in the membrane, a fraction bound via a salt-labile linkage, and a nonextractable fraction; (*c*) the latter two fractions co-localize with actin filament bundles at the basal cell surface; and (*d*) when proteoglycans at the apical cell surface are cross-linked by antibodies, they initially assimilate into detergent-resistant, immobile clusters that are subsequently aggregated by the cytoskeleton. These findings suggest that the proteoglycan, initially present on the entire surface and free in the plane of the membrane, becomes sequestered at the basolateral cell surface and bound to the actin-rich cytoskeleton as the cells become polarized in vitro. Binding of matrix components may cross-link proteoglycans at the basal cell surface and cause them to associate with the actin cytoskeleton, providing a mechanism by which the cell surface proteoglycan acts as a matrix receptor to stabilize the morphology of epithelial sheets.

A central question in cell biology is how cells detect and respond to the extracellular matrix. One potential mechanism involves cell surface matrix receptors which, when occupied by the matrix component, cause the receptor or molecules associated with it to interact with integral membrane or intracellular components. Heparan sulfate proteoglycans at the cell surface have been thought to serve as multivalent matrix receptors (Bernfield et al., 1984a). These molecules bind with varying affinity to several types of matrix components; to interstitial collagens (Stomatoglou and Keller, 1982; Koda and Bernfield, 1984; Koda et al., 1985), to fibronectin (Yamada et al., 1980; Lattera et al., 1983), to laminin (Sakashita et al., 1980), and to other heparan sulfate proteoglycans (Fransson et al., 1983).

Heparan sulfate proteoglycans are present at the cell surface in two apparently distinct forms (Kraemer, 1979; Hook et al., 1983). One class can be competitively displaced from cells by heparin, which indicates that these proteoglycans bind via their heparan sulfate chains to the cell surface (Kraemer, 1977; Kjellan et al., 1980). The other class contains a lipophilic core protein that appears to be integral to the plasma membrane (Kjellan et al., 1981; Norling et al., 1981; Rapraeger and Bernfield, 1983) and has all of its glycosaminoglycan (GAG)[1] chains exposed to the extracellular milieu (Rapraeger and Bernfield, 1985). The former class of proteoglycan is not present on all cell types and has not been extensively studied. The latter class appears to be on all adherent cells, but the proteoglycans vary in size, carbohydrate, and protein composition (Hook et al., 1984).

Matrix receptors should be located at the site where the cell associates with the matrix. Fibroblasts, cells that migrate through connective tissues, must continually make and break their association with the matrix. These cells show heparan sulfate proteoglycans in their substratum adhesion sites (Lark and Culp, 1984) where they co-localize with actin-containing filaments (Laterra et al., 1983). Heparan sulfate proteoglycans also align with actin bundles along the length of fully adherent fibroblasts and coincide with concentrations of actin when the cells are spreading or are rounded (Woods et al., 1984). These localizations are consistent with the finding that lipophilic proteoglycan is retained in the matrix- and cytoskeleton-rich residue that re-

1. *Abbreviations used in this paper*: CMF, Ca^{++}/Mg^{++}-free; GAG, glycosaminoglycan; NMuMG, normal murine mammary gland; TAS, Tris-acetate-buffered saline; TBS, Tris-buffered saline.

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mains after detergent extraction of fibroblasts (Woods et al., 1985) and with the report that lipophilic heparan sulfate proteoglycan binds directly or indirectly to F-actin (Rapraeger and Bernfield, 1982).

On epithelial cells, which form highly polarized sheets or tubes, matrix receptors must be on the basal surface because only this surface abuts the matrix. Unlike fibroblasts, epithelial cells generally do not move in relation to their matrix. Rather, these cells have stable shapes, are bound together by junctional complexes, and have an actin cytoskeleton network near their basal cell surfaces (Fey et al., 1984). The mechanism by which the stable cellular organization and polarity are established is unclear. Although cultured epithelial cells can form polarized epithelial sheets in the absence of observable matrix (reviewed in Simons and Fuller, 1985), several embryonic tissues lose their normal organization and their polarity, as judged by cytoskeletal disorganization, when the matrix at their basal surfaces is lost or destroyed (Bernfield et al., 1972; Hay, 1985).

Normal murine mammary gland (NMuMG) mouse mammary epithelial cells form highly polarized epithelial sheets in monolayer culture (Owens et al., 1974) and can form ductlike structures in vivo (David et al., 1981) and in vitro (Bernfield et al., 1984b). These cells contain a lipophilic cell surface heparan sulfate–rich proteoglycan that binds matrix components with high specificity and affinity (reviewed in Bernfield et al., 1984b). We now show that this proteoglycan has other characteristics of a matrix receptor. All of the proteoglycan is bound to the cell, both by an association with membrane lipid and a fraction linked to components in the detergent-extracted residue; when cross-linked by antibodies at the apical cell surface, the proteoglycan is assimilated into clusters, acquires resistance to detergent extraction, and is aggregated by the cytoskeleton in the plane of the membrane. When the cells are maintained in confluent culture, the proteoglycan is lost from the apical cell surface and localizes solely at the basolateral surface. In these polarized cells, the proteoglycan co-localizes with actin filament bundles at the basal cell surface. These data suggest that this cell surface proteoglycan links the extracellular matrix to the cytoskeleton, and may thus both stabilize epithelial morphology and organize the matrix.

Materials and Methods

Cell Culture and Radiolabeling

NMuMG mouse mammary epithelial cells (passages 13–22) were maintained in bicarbonate-buffered Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) as described previously (David and Bernfield, 1979). For immunochemical experiments, cells were plated at one-quarter confluent density on glass coverslips in 35-mm bacteriological plastic dishes or on 30-mm nitrocellulose filters (Millicel, 0.45 μM; Millipore Corp., Bedford, MA) and grown to 50% of confluent density or grown to confluence and maintained at confluence for one week. For biochemical experiments, cells were plated at one-quarter confluent density on 35-mm tissue culture plastic dishes (Falcon Labware, Oxnard, CA), grown to confluence for 2–3 d or 2 wk, and radiolabeled for 24 h in low sulfate medium containing 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 100 μCi/ml $^{35}SO_4$ (ICN).

Harvest of Radiolabeled Proteoglycan

Assessment of Apical-Basolateral Distribution by Treatment with Trypsin. Radiolabeled monolayers confluent for 2 wk were washed twice in cold Tris-buffered (10 mM, pH 7.4; Sigma Chemical Co., St. Louis, MO) isotonic saline containing 1.25 mM $CaCl_2$ and 0.9 mM $MgSO_4$ (TBS) and incubated (10 min, 4°C) in TBS with or without 20 μg/ml bovine pancreatic trypsin (2× crystallized, type III; Sigma Chemical Co.), followed by addition of soybean trypsin inhibitor (100 μg/ml, type 1-S; Sigma Chemical Co.). The incubation medium was removed, the monolayers were washed once in the cold, and suspended by scraping in cold Ca^{++}/Mg^{++}-free (CMF) TBS containing 0.5 mM EDTA to expose basolateral cell surfaces. After an additional wash and centrifugation (200 *g*), the cells were incubated (10 min, 4°C) in CMF-TBS containing 0.5 mM EDTA with or without 20 μg/ml trypsin. Soybean trypsin inhibitor was added and the cells centrifuged again to obtain the proteoglycan released into the supernatant. Trypsin solutions that had been used for treatment of apical surfaces or suspended cells were shown to retain full activity, as assessed by hydrolysis of *p*-tosylargininemethylester.

Removal of cell surface proteoglycan by treatment with trypsin was also assessed by immunochemistry (Fig. 1). Monolayers were incubated (10 min, 37°C) in CMF-PBS (10 mM, pH 7.4) containing 0.5 mM EDTA to cause cell rounding. The cells were then incubated (10 min, 4°C) in CMF-PBS containing EDTA with or without trypsin (20 μg/ml), treated with soybean trypsin inhibitor (100 μg/ml) and washed in PBS before fixation and immunostaining.

Extraction of Cellular Proteoglycan with Detergents. To quantify the extraction of proteoglycan by saponin or Triton X-100, radiolabeled monolayers confluent for 2–3 d were washed twice in TBS (4°C), then scraped and washed twice in CMF-TBS containing EDTA. The cells were suspended in Tris-acetate-buffered saline (TAS; 10 mM Tris, 50 mM sodium acetate, 150 mM sodium chloride, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, 5 mM benzamidine) adjusted to pH 5.0 or 7.5 with acetic acid or sodium hydroxide, and containing either (*a*) 5 mM saponin (Fisher Scientific Co., Pittsburgh, PA) and 0.5 M KCl, (*b*) 1% Triton X-100, or (*c*) 1% Triton X-100 and 0.5 M KCl. When sequential extractions were performed (cf. Fig. 4), the extracted residue was centrifuged (1000 *g*, 5 min) and resuspended in the subsequent extraction mixture. The final residue was extracted overnight at −20°C in acetate-buffered (50 mM, pH 5.8) 4 M guanidinium chloride containing 5 mM EDTA and 5 mM benzamidine.

Extraction of proteoglycan was also assessed by immunochemistry. Subconfluent monolayers were extracted in TAS (pH 5.0 or 7.5) containing 1% Triton X-100 for 10 min on ice either before or after incubation with antibodies and then fixed. The effect of prior GAG removal on proteoglycan extraction was determined by incubating (37°C, 20 min) monolayers before detergent extraction with chondroitin sulfate ABC lyase (ABCase, 0.05 U/ml, Miles Laboratories, Inc., Naperville, IL) and heparan sulfate lyase (heparitinase, 0.5 U/ml, Miles Laboratories, Inc.) in PBS containing 1 mg/ml bovine serum albumin. Activity of the enzymes was verified by monitoring radiolabel release from monolayers labeled with $^{35}SO_4$. Enzyme concentrations appropriate for this incubation were determined by incubating suspended radiolabeled cells with a range of enzyme concentrations and comparing $^{35}SO_4$ GAG release with that obtained with trypsin (described in Rapraeger and Bernfield, 1985). The enzyme concentrations used here released >90% of the cell surface GAG during the 20-min incubation.

Quantification of $^{35}SO_4$-labeled Proteoglycan

Cetylpyridinium Chloride–TCA-treated Filters. As described in detail elsewhere (Rapraeger and Bernfield, 1985), proteoglycan is quantitatively retained as the sole radiolabeled material when $^{35}SO_4$-labeled cells, detergent, or trypsin-released materials are applied to cetylpyridinium chloride–impregnated filter (3MM; Whatmann, Inc., Clifton, NJ) discs and the discs washed sequentially in 20 mM sodium sulfate, 20% trichloroacetic acid, and 95% ethanol.

Sepharose CL-4B Chromotography. Size exclusion chromatography was performed at room temperature on a column (0.8 × 50 cm) of Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) eluted at 0.1 ml/min, as described previously (Rapraeger and Bernfield, 1985). Samples were eluted in 4 M guanidine HCl containing 5 mM EDTA, 5 mM benzamidine, and 1% Triton X-100 and monitored by a continuous flow radioactivity detector (ISOFLO; Nuclear Enterprises America, Fairfield, NJ). FITC-conjugated dextran 2,000 and fluorescamine-labeled ethanolamine were used as void and total volume markers, detected by a fluorescence monitor (model 200; Waters Instruments, Inc., Rochester, MN).

Immunostaining

Antibodies. Immunolocalization was done with a rat monoclonal IgG2A (mAb 281) specific for the core protein of the cell surface heparan sulfate–rich proteoglycan of the NMuMG cells (Jalkanen et al., 1985). mAb 281 was used in PBS at 50 μg/ml in the presence of 1 mg/ml nonspecific

rabbit or goat serum IgG (Cappel Laboratories, Cochranville, PA). Control stainings, which were negative, used 50 μg/ml of either a nonspecific rat serum IgG or a rat monoclonal IgG2A (kindly supplied by Dr. Sirpa Jalkanen, Department of Pathology, Stanford University School of Medicine) generated by fusion of rat spleen cells with the same myeloma cell line (SP2/0) as mAb 281, but specific for lymphocyte homing receptor. Bound antibodies were localized by FITC-conjugated rabbit anti-rat IgG (1:50; Dako Corp., Santa Barbara, CA) or a TRITC-conjugated goat anti-rat IgG (1:20; Jackson Immuno Research Laboratories, Inc., Avondale, PA).

Staining Living Cells. Living cells (Figs. 1-3, 7, and 8) were incubated with mAb 281 for 30 min and washed five times with PBS on ice, then incubated for 30 min with FITC- or TRITC-conjugated anti-rat IgG and washed five times in PBS. Cells were fixed by sequential treatment with acetone on ice (50%, 1 min; 100%, 5 min; 50%, 1 min) followed by three washes in PBS. After fixation, selected TRITC-labeled monolayers were incubated with FITC-conjugated phalloidin (1:20 in PBS; Molecular Probes, Inc., Junction City, OR) for 30 min to localize filamentous actin. After a fivefold PBS wash, the coverslips were mounted on glass slides in Immumount (Shandon Southern Instruments, Inc., Sewickley, PA) and viewed on a Zeiss Photomicroscope II equipped with epifluorescence. Pictures were recorded on Kodak Tri-X film exposed at ASA 1,000.

Staining Cells on Nitrocellulose Filters. Cells grown at confluence for 1 wk on nitrocellulose filters (Fig. 2, *E* and *F*) were incubated for 30 min on ice with mAb 281 in TBS containing 10% serum, washed five times for 10 min in TBS/10% serum, incubated for 30 min in TRITC-goat anti-rat IgG, and finally washed five times for 10 min in TBS/10% serum. The filters were fixed overnight in 4% formaldehyde in PBS (4°C), incubated 1 h at room temperature in 50 mM ammonium chloride, then embedded in Eukitt mounting medium (Calibrated Instruments, Inc., Ardsley, NY) after dehydration in ethanol.

Staining Detergent-extracted Residues. Monolayers were incubated (30 min, 37°C) in culture medium with or without cytochalasin D (final concentration, 2 μM in 1% DMSO), then extracted (10 min, 4°C) in TAS (pH 7.5) containing 1% Triton X-100 (see Figs. 5 and 6). The residues were washed three times in PBS, fixed in 4% formaldehyde on ice (30 min) followed by incubation in 50 mM ammonium chloride in PBS (30 min), and sequential treatment with 50, 100, and 50% acetone, then stained with mAb 281 and phalloidin as described above.

Results

Distribution of Cell Surface Proteoglycan on Mammary Epithelial Cells

Mammary epithelial cells abut the extracellular matrix at their basal surfaces, whereas their apical surfaces are exposed to the acinar or ductal lumen. Because the cell surface proteoglycan has properties of a matrix receptor, its distribution on the surface of cells cultured in vitro was investigated.

Proteoglycan is Localized on the Surface of NMuMG Cells by a Monoclonal Antibody. Subconfluent monolayers of NMuMG mouse mammary epithelial cells contain islands of cells; in the center of these islands the cells are contiguous (Fig. 1 B), whereas cells at the borders have a free margin. Immunolocalization of cell surface proteoglycan in these cultures using a monoclonal antibody (mAb 281), which binds specifically to the proteoglycan core protein, shows staining of the cell surface (see also Jalkanen et al., 1985). The stain is particularly evident where the cells contact one another (Fig. 1 *A*) and examination at various focal planes shows stain on the apical surface, but none is apparent at the basal surface.

When the cells are stained after brief incubation in warm EDTA, which causes them to round, cell surface proteoglycan surrounds the cells (Fig. 1 *C*), possibly due to redistribu-

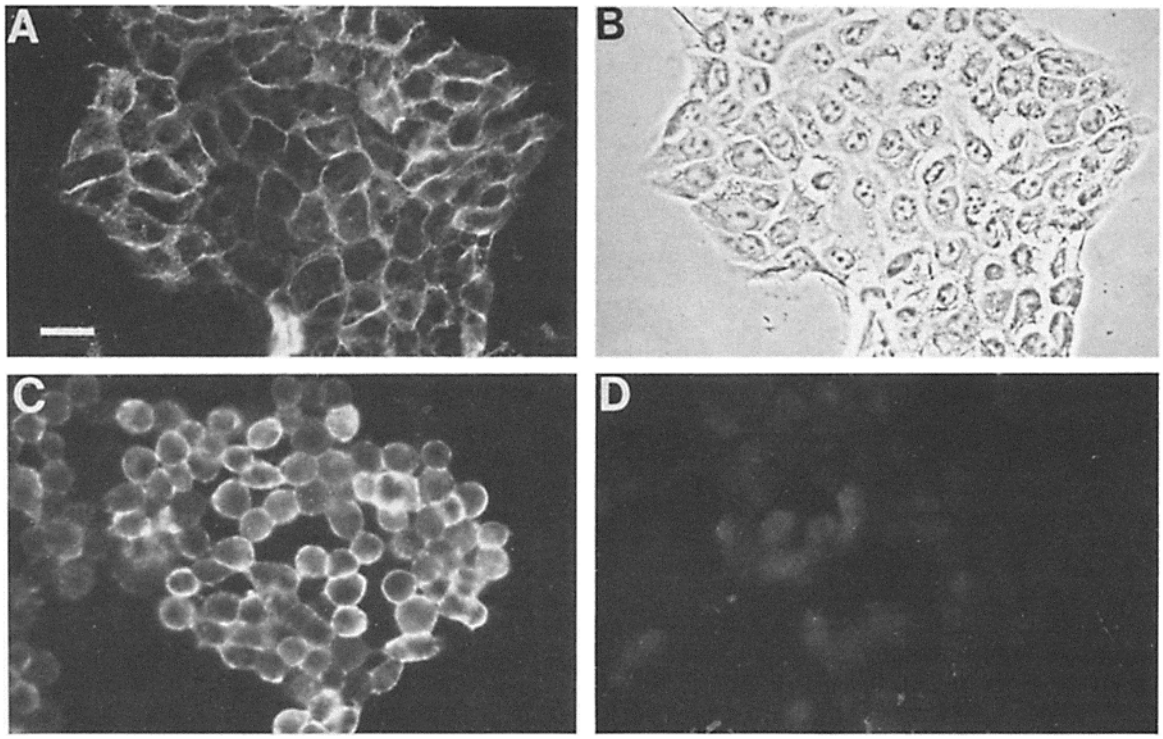

Figure 1. Localization of cell surface proteoglycan on subconfluent epithelial cells. Cells were incubated in PBS with or without 0.5 mM EDTA for 10 min at 37°C, followed by incubation for 10 min in PBS at 4°C. Some cells (*D*) were exposed to trypsin (20 μg/ml, 10 min, 4°C) during the latter incubation, followed by soybean trypsin inhibitor (100 μg/ml). The cells were incubated with mAb 281, fixed, then incubated with an FITC-conjugated anti-rat IgG. Substitution of a nonspecific rat IgG for mAb 281 results in no staining (not shown). (*A* and *B*) immunofluorescent (*A*) and phase-contrast (*B*) photographs of cells not treated with EDTA. (*C* and *D*) immunofluorescent photographs of cells stained after treatment with EDTA (*C*) or EDTA followed by trypsin (*D*). Bar, 20 μm.

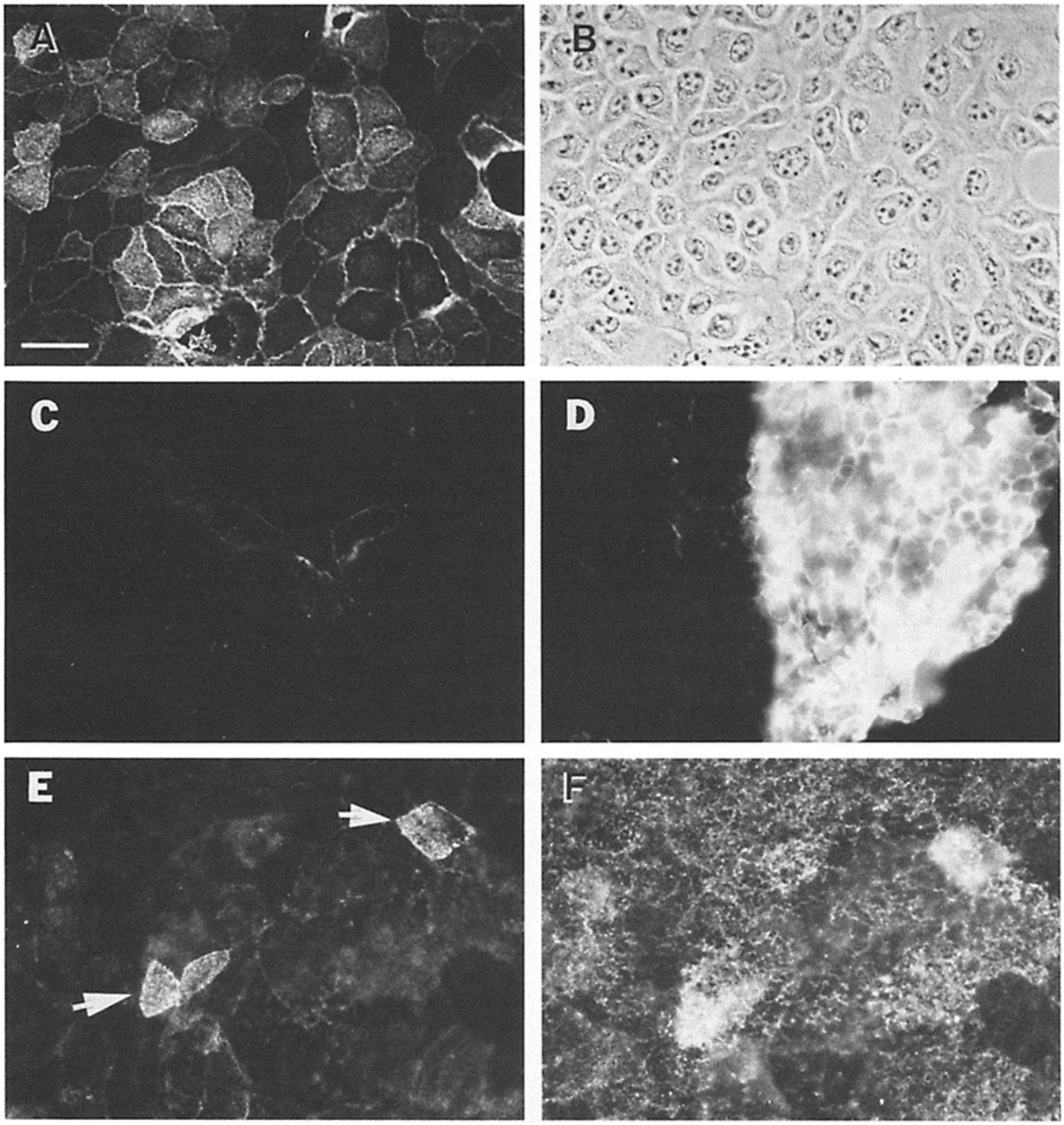

Figure 2. Localization of cell surface proteoglycan on epithelial monolayers confluent for 1 d or 2 wk. Monolayers were cultured at confluence for 1 d or 1 wk on tissue culture plastic (*A–D*) or on nitrocellulose filters for one week (*E* and *F*). For detecting apical and basolateral cell surface proteoglycan, the monolayers were incubated in PBS with or without 0.5 mM EDTA for 10 min at 37°C, then incubated with mAb 281 followed by a fluorescent-conjugated anti-rat IgG and fixed (see Materials and Methods). (*A* and *B*) Immunofluorescent (*A*) and phase-contrast (*B*) photographs of a monolayer grown at confluence for 1 d on tissue culture plastic. (*C* and *D*) Immunofluorescent photography of monolayers grown at confluence for 1 wk and treated with (*D*) or without (*C*) EDTA before antibody staining. The field in *C* was selected to show rare cells exhibiting apical stain. In *D*, the field shows a portion of a monolayer that has been detached by the EDTA, a common result in monolayers confluent for a week or more, and has folded back on itself (determined by observing various focal planes) to simultaneously display both basal (bright stain) and apical cell surfaces. (*E* and *F*) Immunofluorescent photography of a monolayer grown at confluence for 1 wk on a nitrocellulose filter to allow antibody access to the basolateral surface without disturbing the monolayer. *E* shows a few rare cells (*arrows*) that exhibit stain seen in a focal plane at their apical surfaces. In addition, some faint lateral staining is seen and basal staining that is not in focus. *F* shows the identical portion of the monolayer, but a focal plane at the basal cell surface. Stain is seen at the basal surface of almost all the cells. The apical stain observed in *E* is now out of focus and appears as a blur. Bar, 30 μm.

tion in the plane of the membrane upon cell rounding. Mild treatment of these cells with trypsin (20 μg/ml, 5 min, 4°C), abolishes the staining, consistent with previous work showing that this treatment releases the proteoglycan's extracellular domain (ectodomain) that bears the mAb 281 antigenic site (Rapraeger and Bernfield, 1985; Jalkanen et al., 1985).

Proteoglycan Becomes Sequestered at the Basolateral Cell Surface in Confluent Monolayers. When monolayers that have been confluent for a day are stained with mAb 281, staining of the cell surfaces is not uniform; some cells show apical and lateral staining, others show reduced stain at their lateral borders, and some cells show none (Fig. 2 *A*). The number of cells showing no stain is dramatically increased when cells are maintained at confluence for a week or more. Staining is seen only on a few isolated cells, often present in small groups, and appears to be solely on the apical cell surface (Fig. 2 *C*). This suggests that with time at confluence either the proteoglycan is no longer expressed at the cell surface of most cells or it is confined to the basolateral surface where it is not seen because the development of lateral cell junctions prevents access of antibodies to these surfaces.

To quantify the relative amounts of proteoglycan at the apical and basolateral cell surfaces, monolayers were cultured for two weeks postconfluence on plastic, then labeled with $^{35}SO_4$. The radiolabeled cell surface proteoglycan was removed with trypsin (20 μg/ml, 10 min, 4°C) either from the apical surfaces or, after cell rounding in EDTA, from the entire cell surface (cf. Figs. 1 *C* and *D*). Of the total cell surface proteoglycan released (131,912 cpm/dish [±3.1% SEM, $n = 5$]), only 4.2% (0.3% SEM, $n = 5$) is released from the apical surfaces, indicating that almost all of the proteoglycan in these cultures is at the basolateral surface.

This finding was corroborated by immunolocalization studies using cultures confluent for 1 wk in which mAb 281 was allowed access to basolateral cell surfaces. Access was provided either by EDTA treatment of monolayers grown on coverslips, which detaches portions of the monolayer, or by culture of the cells on nitrocellulose filters, which allows mAb 281 access via the filter pores without disturbing the monolayer. After treatment of the confluent monolayers with EDTA and staining with mAb 281, bright stain is seen where portions of the monolayer are detached and flipped over, exposing the basal surface (Fig. 2 *D*). In contrast, the apical surface remains unstained. A similar finding is seen when identical cultures are examined *en face* on nitrocellulose filters, where staining with mAb 281 detects apical proteoglycan on only a few rare cells (see arrows in Fig. 2 *E*) demonstrating stained cell surfaces in the apical focal plane). However, observation of a narrow focal plane corresponding to the basal cell surface shows a fibrous staining pattern for almost all the cells. Lateral stain is only slightly evident, although this is variable and may depend on accessibility of the antibody into the lateral cell space. The stain is not seen throughout the filter itself and is abolished by mild treatment with trypsin, suggesting that the antigen is anchored at the cell surface. Therefore, although proteoglycan is at the apical surface of subconfluent and newly confluent monolayers, confluent monolayers cultured for a week or more lose this apical proteoglycan and sequester the molecule almost solely on their basolateral cell surfaces.

Table I. Effect of pH and KCl on Extraction of Cell Surface Proteoglycan by Triton X-100

Extraction conditions*	Cell surface proteoglycan released
	%
pH 7.5	67.3 (± 1.3, $n = 6$)
pH 5.0	<5 ($n = 3$)
pH 7.5 + 0.5 M KCl	78.9 (± 2.9, $n = 3$)
pH 5.0 + 0.5 M KCl	79.6 (± 1.7, $n = 3$)

* Monolayers labeled with $^{35}SO_4$ for 24 h were scraped in EDTA, washed, then extracted in TAS + 1% Triton X-100 at pH 7.5 or 5.0 and with or without 0.5 M KCl. Proteoglycan released by a pretreatment of the suspended cells with 20 μg/ml trypsin (10 min, 4°C) is defined as the cell surface form (Rapraeger and Bernfield, 1985).

Anchorage of Proteoglycan to the Cell

One explanation for the presence of proteoglycan on the apical surface only in subconfluent or newly confluent monolayers is that it escapes from the basolateral cell surface by diffusion in the plane of the membrane, presumably because the cell junctions that seal apical from basolateral membrane domains are not complete. This hypothesis requires that the proteoglycan be a mobile, integral membrane protein, as has been suggested previously (Rapraeger and Bernfield, 1983). Therefore, its susceptibility to extraction with detergents was explored to determine the nature of its association with the cell.

Extraction of Cell Surface Proteoglycan with Detergents. When $^{35}SO_4$-labeled NMuMG cells are suspended at 4°C by scraping in EDTA and are washed by centrifugation, a proteoglycan remains on the surface of the suspended cells. This proteoglycan is displaced from intact cells only by proteolytic cleavage (Rapraeger and Bernfield, 1985); it is not displaced by treatment with 100 μg/ml heparin (Rapraeger and Bernfield, 1985), removal of its GAG chains (see below), or washing with 1.5 M KCl (see below). Extraction of the suspended cells with 1% Triton X-100 in an isotonic saline (TAS, pH 7.5), however, releases 67.3% of the cell surface proteoglycan, as assessed by comparing cells treated with or without trypsin before the extraction (Table I). The remainder sediments with the Triton-insoluble cell residue. Repeated washing of this residue with Triton fails to remove more proteoglycan, suggesting that the cell surface molecule partitions between a class that is susceptible to extraction, presumably free in the membrane, and a class anchored to insoluble elements, possibly components of the intracellular cytoskeleton.

The proportion of Triton-insoluble cell surface proteoglycan increases when the pH of the extraction mixture is reduced. When the pH is decreased to 5.0, >95% of the cell surface proteoglycan becomes resistant to extraction (Table I). This enhanced association with the cell residue is reversible, as it is abolished by a subsequent wash with Triton at pH 7.5. Substitution of 75 mM octylglucopyranoside, another nonionic detergent, in place of Triton X-100 yields identical results, suggesting that the pH change affects the associations of the proteoglycan rather than the action of the detergent.

The pH-dependent extraction of cell surface proteoglycan by Triton X-100 is confirmed by indirect immunofluorescent

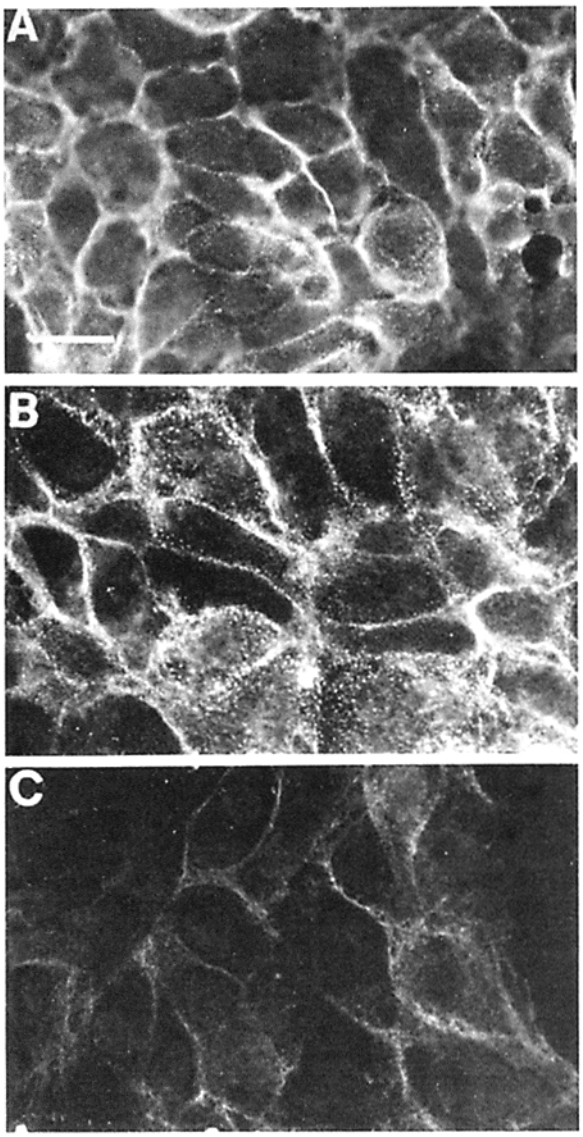

Figure 3. Extraction of GAG-free apical cell surface proteoglycan with Triton X-100. Subconfluent monolayers were treated with heparitinase and ABCase to remove the GAG chains from the apical cell surface proteoglycan. The cells were extensively washed and incubated at 4°C with mAb 281. The cells were then extracted with (*A*) TAS (pH 7.5) without detergent, (*B*) TAS (pH 5.0) containing 1% Triton X-100, or (*C*) TAS (pH 7.5) containing 1% Triton X-100. The residues were fixed in TAS at the extraction pH according to the procedure described in Materials and Methods, followed by incubation with a second, FITC-conjugated antibody. Photographs were taken with identical time exposures. Bar, 20 μm.

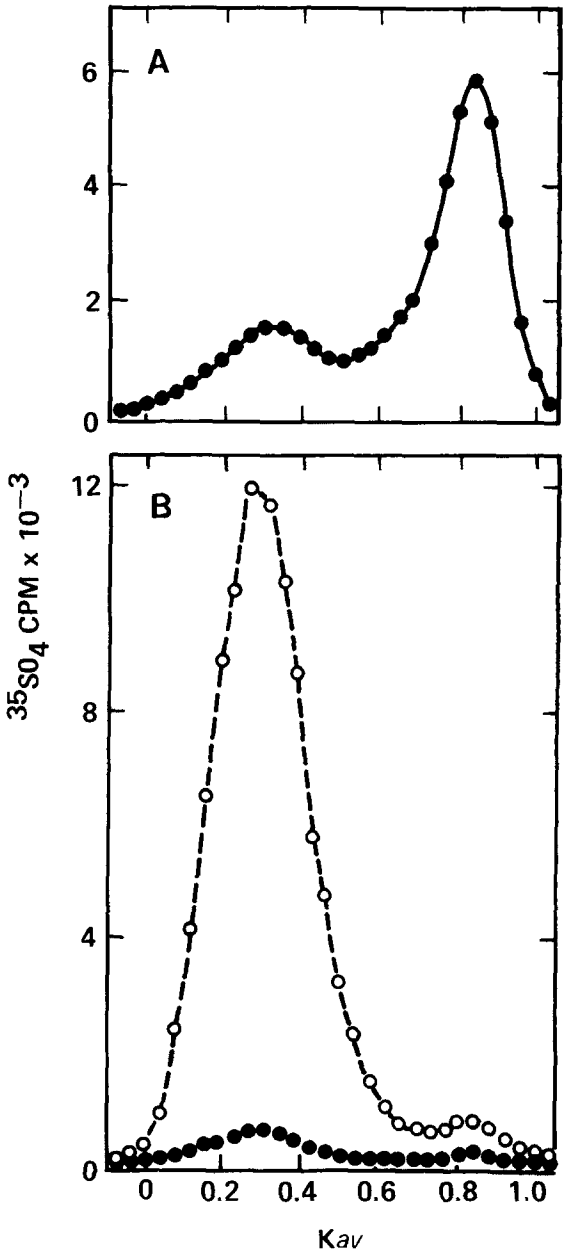

Figure 4. Sepharose CL-4B analysis of $^{35}SO_4$-labeled material extracted by sequential treatments with saponin and KCl; Triton X-100; and Triton X-100 and KCl. Confluent monolayers were radiolabeled and scraped, then extracted in TAS (pH 5.0) containing 2 μg/ml saponin and 0.5 M KCl. The residue from this treatment was further extracted in TAS (pH 5.0) containing 1% Triton X-100 and, lastly, extracted in TAS (pH 5.0) containing 1% Triton X-100 and 0.5 M KCl. The $^{35}SO_4$-labeled materials released by each of these treatments were precipitated in ethanol, then chromatographed on Sepharose CL-4B in 1% sodium dodecyl sulfate. (*A*) $^{35}SO_4$-labeled materials released by saponin plus 0.5 M KCl. (*B*) $^{35}SO_4$-labeled materials released by Triton alone (*solid circle*) or Triton containing 0.5 M KCl (*open circle*).

staining of monolayers using mAb 281 (Fig. 3). As described above, this staining method primarily localizes proteoglycan on the apical cell surface. Living, confluent monolayers were treated with the antibody to localize proteoglycan solely at the cell surface, then extracted with Triton at pH 7.5 or 5.0. In addition, in an attempt to rule out potential binding of the proteoglycan to extracellular matrix materials via its GAG chains, the monolayers were pretreated with heparitinase and chondroitin sulfate ABCase. Release of the chains was confirmed by the release of $^{35}SO_4$-labeled GAG fragments into the medium. When such cells are simply washed, then fixed and immunostained, the core protein is identified primarily at the cell margins but also in a faint, sometimes punctate, pattern on the apical surface of some cells (Fig. 3 *A*). If the cells are extracted with Triton X-100 at pH 5.0, fixed at this pH, and stained, little effect is seen on the amount of stain-

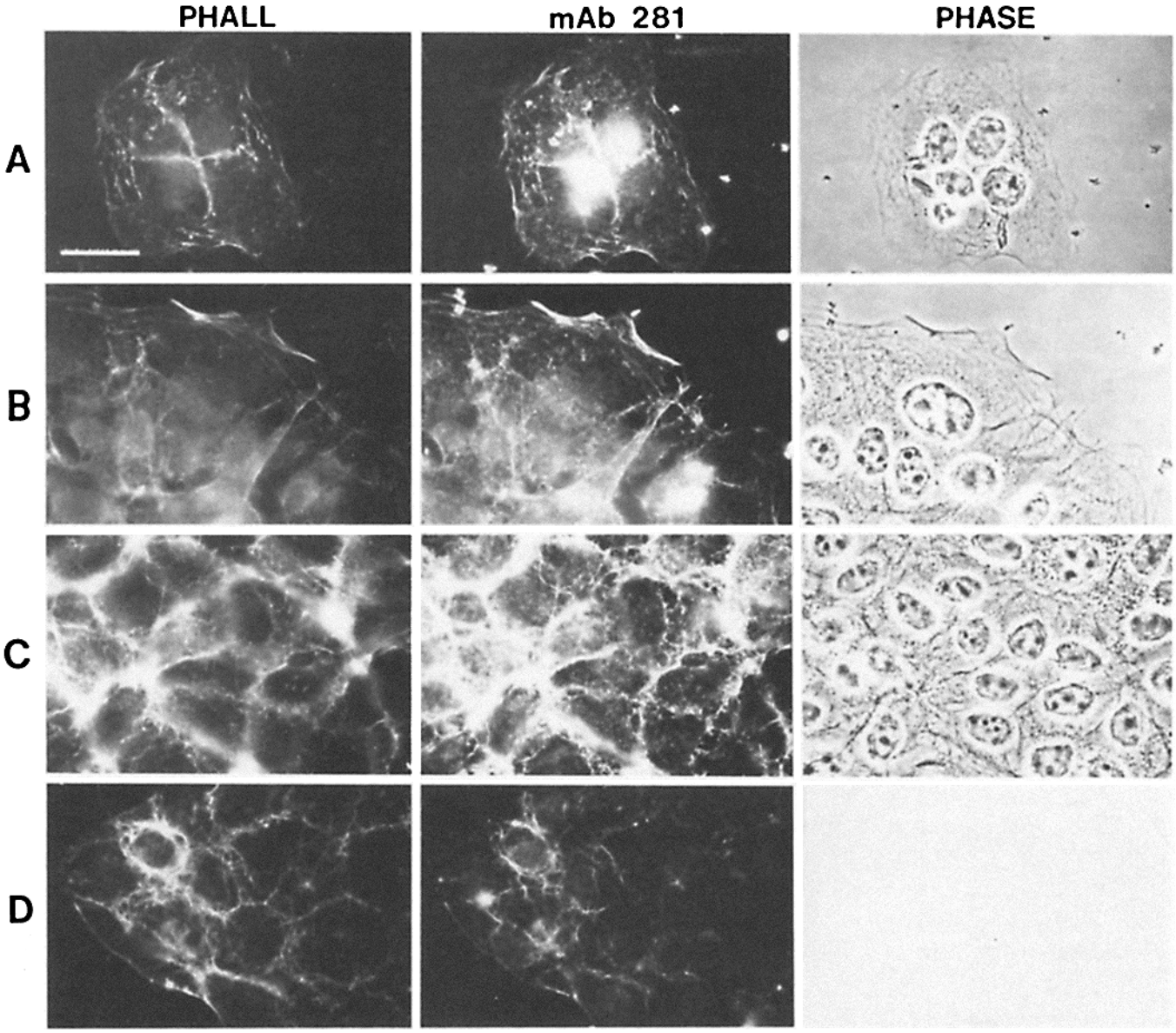

Figure 5. Localization of proteoglycan and filamentous actin in subconfluent cells after extraction with Triton X-100. Subconfluent monolayers were extracted in TAS (pH 7.5) containing 1% Triton X-100 (*A–C*) or containing 1% Triton X-100 and 0.5 M KCl (*D*). The residues were fixed, then incubated with mAb 281 followed by a TRITC-conjugated anti-rat IgG. The residues were also incubated with FITC-conjugated phalloidin to detect filamentous actin. Panels are immunofluorescent photographs of FITC-conjugated phalloidin (PHALL) or the TRITC-conjugated antibody (mAb 281) and phase-contrast photographs of the Triton-extracted residues (PHASE). (*A*) Group of four cells; (*B*) spreading margin of an island of cells; (*C*) confluent cells in the center of a large island of cells; and (*D*) spreading margin of cell island extracted with KCl in addition to Triton X-100. Bar, 10 μm.

ing, although the pattern over the apical surface appears more punctate (Fig. 3 *B*). Staining of apparently identical intensity and distribution is seen in the absence of heparitinase and ABCase pretreatment, suggesting that the resistance of the proteoglycan to extraction is not due to binding of its GAG chains. Extraction at pH 7.5, as expected from the quantification studies (cf. Table I), substantially reduced the amount of staining; the remaining core protein is in faint filamentous arrays predominantly at the cell margins (Fig. 3 *C*).

Triton-resistant Cell Surface Proteoglycan Is Bound by a Salt-labile Linkage. Treatment of Triton-extracted residues with TAS supplemented with KCl releases additional proteoglycan, with maximal release occurring at an added KCl concentration of 0.5 M or greater. This effect is most striking with cells extracted at pH 5.0; whereas the Triton treatment alone releases little cell surface proteoglycan, increasing the salt concentration of the extraction mixture by adding 0.5 M KCl releases 79.6% (Table I). A lesser effect is seen at pH 7.5 because the bulk of the cell surface proteoglycan is susceptible to the initial extraction; 78.9% is released in the presence of added KCl compared with 67.3% released in its absence. These data suggest that ~80% of the cell surface proteoglycan is released from the cell by nonionic detergents in a pH- and salt-dependent manner. The remaining 20% appears to be more stably anchored.

In an attempt to pinpoint the salt-labile site either at the surface of the cell or in the membrane, $^{35}SO_4$-labeled cells were treated with KCl (*a*) before detergent extraction; (*b*) after permeabilization with saponin, a detergent that disrupts cholesterol-rich areas of the membrane (Seeman, 1967); and (*c*) after treatment with Triton. Treatment of suspended cells with up to 1.5 M KCl in TAS without detergent failed to release proteoglycan, regardless of pH. When the cells were treated with saponin containing 0.5 M KCl at pH 5.0, radiolabeled products were released and identified as proteoglycan or intracellular GAG fragments as previously defined (Rapraeger and Bernfield, 1985). Quantification after chromatography on Sepharose CL-4B (Fig. 4 *A*) demonstrates that 24% of the cellular proteoglycan (K_{av} of 0.33) is released by saponin/KCl together with most of the GAG fragments (K_{av} of 0.85), indicating that saponin treatment permeabilized the cell, providing the KCl with access to the cytoplasm.

The effect of pH 5.0 saponin plus KCl is apparently the same as that of a pH 5.0 Triton treatment alone because (*a*) identical amounts and types of products are extracted by TAS (pH 5.0)/1% Triton X-100 (not shown) and (*b*) almost no additional materials are released by this treatment from residues previously extracted by saponin/KCl (Fig. 4 *B*). The proteoglycan fraction released is not the cell surface form and apparently corresponds to the intracellular, nonlipophilic fraction described previously (Rapraeger and Bernfield, 1985). Cell surface proteoglycan is released only after treatment of the saponin–KCl-extracted residues with TAS (pH 5.0)/1% Triton X-100 containing 0.5 M KCl (Fig. 4 *B*). Thus, both disruption of the membrane and high salt are required to extract the cell surface proteoglycan at pH 5. These results support the hypothesis that the proteoglycan is anchored in the plasma membrane and, at pH 5.0, is anchored by ionic interactions, possibly with other proteins in or adjacent to the membrane.

Proteoglycan Colocalizes with F-Actin at the Basal Cell Surface

To assess a potential cytoskeletal linkage for the cell surface proteoglycan fraction(s) that is most stably anchored to the cell, the ~33% that resists detergent extraction at pH 7.5 (cf. Table I) was stained with mAb 281 and compared with the distribution of filamentous actin as detected with fluorescent phalloidin.

Localization in Newly Confluent Monolayers. Extraction and fixation of subconfluent cells or newly confluent monolayers, followed by incubation with mAb 281 and detection by a rhodamine-conjugated anti-rat IgG and simultaneous staining with FITC-conjugated phalloidin shows that actin and proteoglycan co-localize in filamentous arrays in a focal plane corresponding to the basal cell surface. When the cells are in small groups, the proteoglycan is seen together with actin at the lateral cell borders and in the highly spread cell margins where actin fibers run parallel to or intersect the cell borders (Fig. 5 *A*). A similar pattern is seen at the periphery of a large island of cells where actin is present in fibers close to the spread cell margins. Localization of proteoglycan indicates that it is highly coincident with the actin distribution (Fig. 5 *B*). Cells that are newly confluent lack free margins and display only irregular actin filament bundles at their basal surfaces; these bundles also distribute coincidently with the proteoglycan at basal cell surfaces (Fig. 5 *C*).

As demonstrated biochemically, a fraction of the cell surface proteoglycan resists not only Triton extraction, but also release by KCl. Fluorescent detection of F-actin and the proteoglycan is difficult in KCl-treated residues because the KCl treatment displaces the residue from the coverslip. In the few cases where localization has been possible, fluorescence for both F-actin and proteoglycan are seen in the residue, but are reduced in intensity. The residual actin is present in a delicate fibrous array adherent to the substratum and is less evident at the lateral cell borders (Fig. 5 *D*). Staining with mAb 281 indicates that proteoglycan localizes to some of these fibers, although it is lacking in others (Fig. 5 *D*).

Localization after Prolonged Culture of Confluent Monolayers. Localization of F-actin and proteoglycan in monolayers cultured for a week or more demonstrates a network of fibers at the basal cell surface that is not seen in subconfluent or newly confluent cultures. The actin is organized into highly aligned fibers spanning the length of the cell (Fig. 6 *A*). Staining with mAb 281 very nearly duplicates this fibrous network, which can be seen only in a narrow focal plane at the basal surface of the cells (Fig. 6 *A*). A focal plane taken through the level of the nucleus, for example, demonstrates that staining with phalloidin and mAb 281 is largely restricted to the lateral borders of the cells (Fig. 6 *B*). Thus, the polarized distribution of proteoglycan in confluent monolayers is accompanied by (*a*) the alignment of actin into fibers at the basal cell surface and (*b*) co-localization of proteoglycan with these fibers.

Association of the proteoglycan with the actin fibers is suggested by the effect of cytochalasin D on proteoglycan distribution. Treatment with 2 μM cytochalasin D results in the collapse of the basal actin fibers into aggregates (Fig. 6 *C*). Examination of proteoglycan in the cytochalasin-treated cells indicates that it also collapses into discrete aggregates and that these coincide with the actin (Fig. 6 *C*). This staining is specific for mAb 281 as it is not duplicated by nonspecific antibodies (Fig. 6 *D*). In addition, the staining is not due to nonspecific binding of mAb 281 as it fails to stain Triton-extracted residues of bovine aortic endothelial cells that contain copious amounts of actin filaments, detected by phalloidin (not shown).

Cell Surface Proteoglycan Associates with the Cytoskeleton when Cross-linked

Integral membrane proteins on suspended cells aggregate and cap in an energy-dependent manner if cross-linked by exogenous ligands, a behavior apparently mediated by their association with submembranous cytoskeletal components (reviewed by de Petris, 1977 and Bourguignon and Bourguignon, 1984). The co-localization of proteoglycan with actin filament bundles at the basal cell surface could result from extracellular cross-linking of mobile proteoglycan anchored in the basal plasma membrane, promoting an association of the proteoglycan with the cytoskeleton and thereby converting it from a Triton-soluble (pH 7.5) proteoglycan to an insoluble form. To assess whether this mechanism is possible, proteoglycan on the apical cell surface, which is susceptible to Triton extraction at pH 7.5 (cf. Fig. 3 *C*), was cross-linked with antibodies.

Cross-linked Cell Surface Proteoglycan Is Assimilated into Clusters. When cells are treated at 4°C with both mAb 281 and FITC-conjugated anti-rat IgG before fixation, the

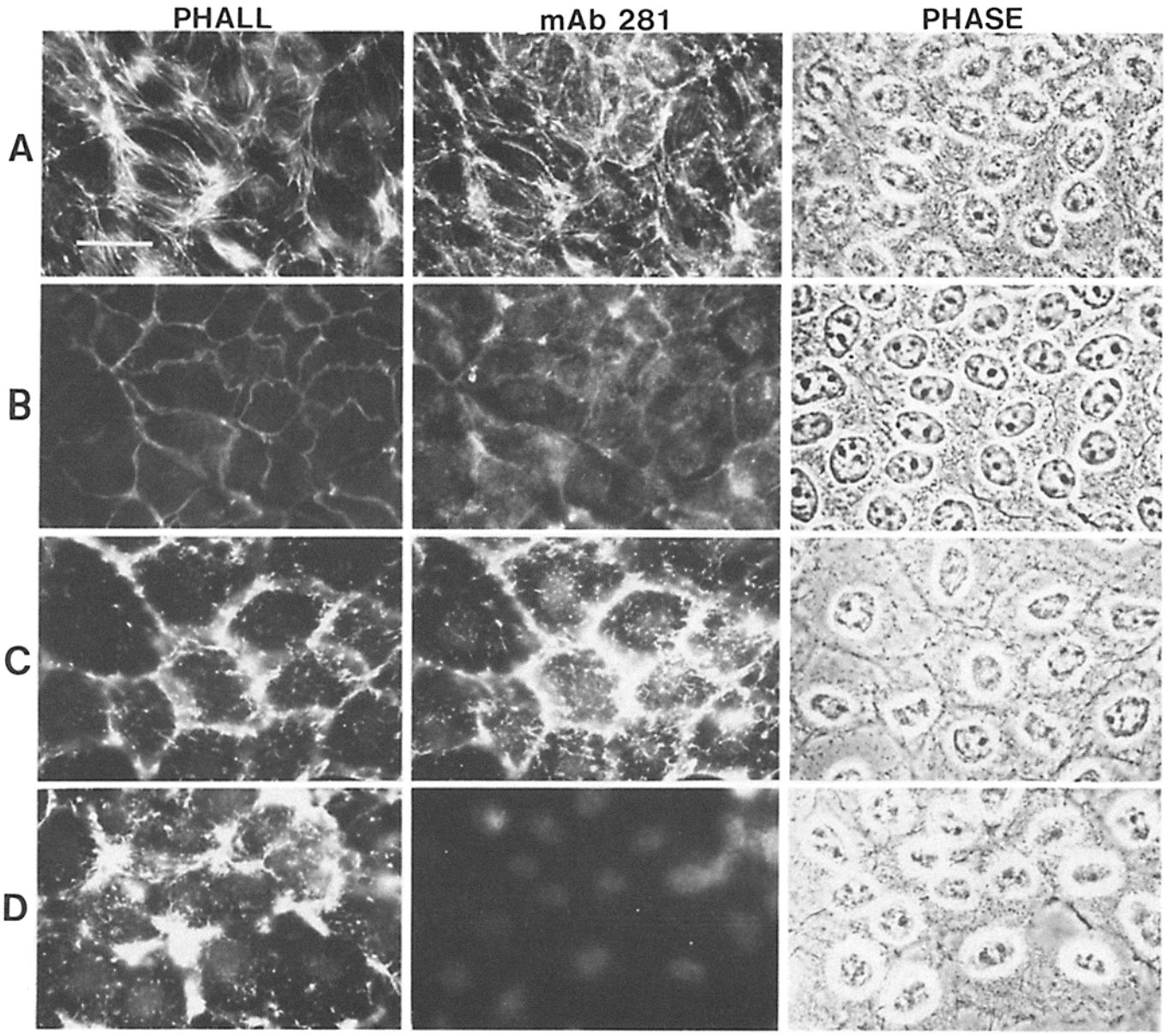

Figure 6. Localization of proteoglycan and filamentous actin after Triton X-100 extraction of monolayers confluent for 1 wk. Monolayers were grown to confluence and then cultured for an additional week. Cytochalasin D (2 μM) was added to the culture medium for 20 min, then the monolayers were extracted with TAS (pH 7.5) containing 1% Triton X-100. After fixation, the residues were labeled with antibodies or phalloidin as described in Fig. 5. Panels are immunofluorescence photographs of FITC-conjugated phalloidin (PHALL) or TRITC-conjugated antibody (mAb 281) or phase-contrast photographs (PHASE) of the Triton-extracted residues. (*A*) No cytochalasin treatment, focal plane at basal cell surface; (*B*) same field as in *A* with focal plane in the middle of the cell; (*C*) treated with cytochalasin D, focal plane at basal cell surface; and (*D*) treated with cytochalasin D, focal plane at basal cell surface, nonspecific rat IgG substituted for mAb 281. Bar, 10 μm.

proteoglycan assimilates into clustered sites (Fig. 7 *A*), which appear as dots of stain, both larger and more uniformly dispersed than when fixed before staining with second antibody (Fig. 7 *B*). This assimilation is apparently energy independent, because it occurs at cold temperatures and in the presence of 50 mM sodium azide (data not shown). However, despite the apparent rapid assimilation into clusters, which occurs even during a 20-min staining procedure on ice, the staining pattern does not change if the cells are maintained at 4°C for an additional hour or more. Formation of the proteoglycan clusters is dependent on cross-linking. The proteoglycan does not form clusters when treated with mAb 281 alone (Fig. 7 *B*), even when followed by incubation for 60 min at 37°C before fixation and immunolocalization. Thus, assimilation into clusters is not due to antibody binding alone, but is dependent on cross-linking.

Cross-linked Proteoglycans Redistribute at 37°C. The proteoglycan clusters at the apical surface alter their distribution when the cells are warmed to 37°C. After 10 min, for example, proteoglycan assimilates into larger-sized aggregates that are uniformly present over the apical surface and line the margins of the cells (Fig. 7 *C*). After an hour incubation at 37°C, aggregates are no longer seen at the cell borders, but appear as numerous, irregularly spaced, bright

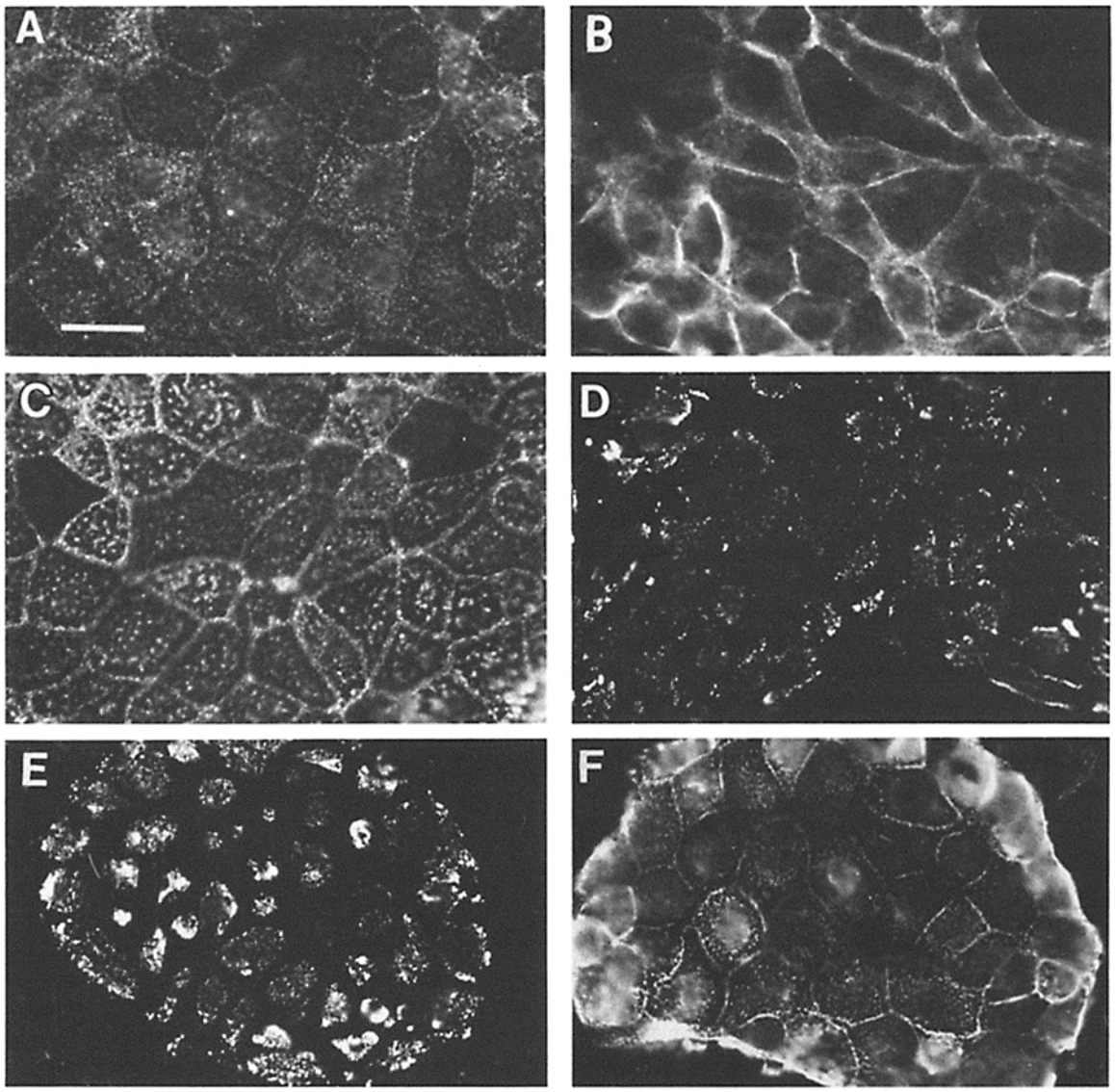

Figure 7. Behavior of apical cell surface proteoglycan cross-linked on living cells with antibodies. Subconfluent monolayers were labeled at 4°C with mAb 281. This was followed immediately by incubation with an FITC-conjugated anti-rat IgG, with the exception of *B*, which was incubated with the monoclonal antibody alone. The monolayers were then incubated at 4°C for 70 min or were incubated for 10 min at 4° in the presence or absence of colchicine (50 μM) or cytochalasin D (2 μM), transferred to 37°C in the presence of these drugs for up to 60 min, then fixed. (*A*) Incubation at 4°C for 70 min; (*B*) incubated at 37°C for 10 min; (*D*) incubation at 37°C for 60 min; (*E*) incubation at 37°C for 60 min in colchicine; and (*F*) Incubation at 37°C for 60 min in cytochalasin D. Bar, 20 μm.

foci (Fig. 7 *D*). In addition, the aggregation appears to be accompanied by a loss of fluorescence, suggesting that the proteoglycan is ultimately shed from the cells. Thus, the behavior of cross-linked proteoglycan is similar to the fate of other integral membrane proteins cross-linked by exogenous ligands (de Petris and Raff, 1973; Nicolson, 1976; de Petris, 1977; Bourguignon and Bourguignon, 1984).

Movement of the Proteoglycan Clusters Is Dependent on the Cytoskeleton. Aggregation and loss of the proteoglycan after cluster formation requires energy and can be affected by cytoskeleton-disrupting agents. Low temperature (cf. Fig. 7 *A*) or 50 mM sodium azide (not shown) treatment of cells bearing cross-linked proteoglycan blocks the aggregation of the clusters. Thus, unlike formation of the clusters, movement of the clusters themselves requires energy.

To identify cytoskeletal components responsible for movement of the proteoglycan clusters, the cells were treated with colchicine or cytochalasin D in an attempt to disrupt microtubules or microfilaments, respectively. Incubation of the cells in 25 μM colchicine has no effect on the movement of the proteoglycan clusters (Fig. 7 *E*). They migrate from the cell borders and appear over the central regions of the cells. However, 2 μM cytochalasin D disrupts the movement of the proteoglycan clusters, which remain in their initial distribution (Fig. 7 *F*). Additionally, in cytochalasin, the clusters do not appear to aggregate further by diffusion, despite incubation at 37°C for up to 4 h. Therefore, once assimilated into clusters, they appear immobilized; any further movement or removal from the cell surface is apparently dependent on the actin-rich cytoskeleton. Occasionally, this cytochalasin treatment causes a collapse of the apical actin architecture, demonstrated by phalloidin staining, resulting in redistribu-

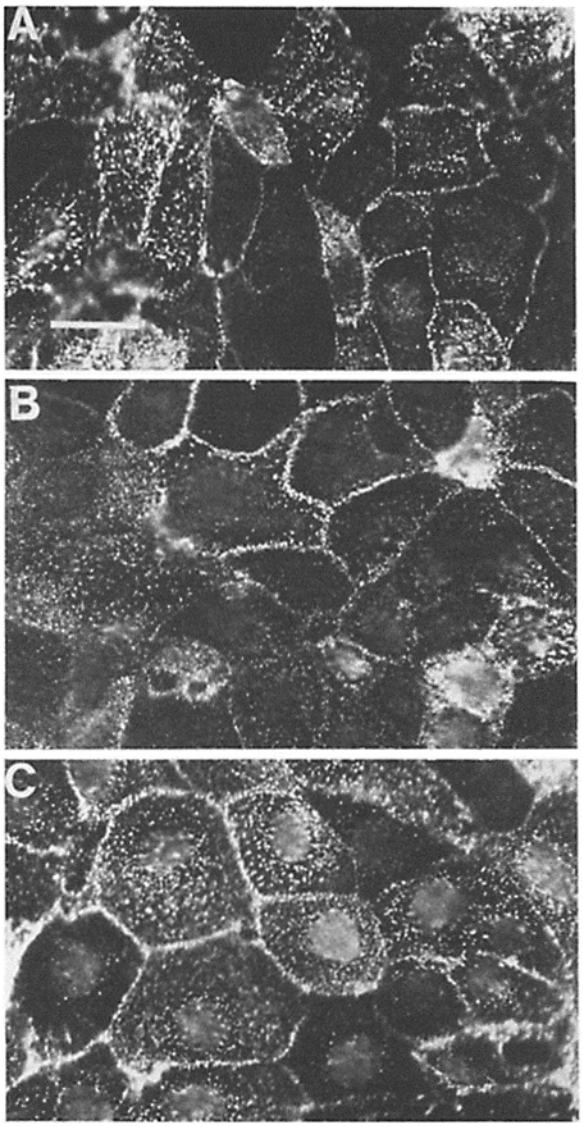

Figure 8. Extraction of apical cell surface proteoglycan with Triton X-100 after cross-linking with antibodies. Subconfluent monolayers were incubated at 4°C with mAb 281 followed by an FITC-conjugated anti-rat IgG. The cells were then extracted with (*A*) TAS (pH 7.5), (*B*) TAS (pH 5.0) containing 1% Triton X-100, or (*C*) TAS (pH 7.5) containing 1% Triton X-100, then fixed. This experiment was done at the same time and with identical photographic techniques as that shown in Fig. 3. Bar, 10 μm.

tion of the actin into several aggregates in the middle of the cell. On these occasions, the proteoglycan also aggregates and co-distributes with the actin. Thus, the proteoglycan may become directly or indirectly associated with the actin-rich cytoskeleton when cross-linked into the clustered sites.

Proteoglycan Clusters Resist Triton Extraction. If the proteoglycan associates with the cytoskeleton after cross-linking into clusters at the cell surface, its extractability with Triton should be altered. To assess this possibility, the cells were incubated with mAb 281 and the second anti-rat IgG before extraction with Triton X-100. The cell surface proteoglycan, now in clusters, is not extracted; no difference in the amount or pattern of staining is detected if the cells are treated with TAS lacking detergent (Fig. 8 *A*) or containing Triton X-100 at pH 5.0 (Fig. 8 *B*) or pH 7.5 (Fig. 8 *C*). Removal of the GAG chains does not influence this behavior (not shown). Thus, it appears that the apical proteoglycan can be induced to associate with the cytoskeleton by cross-linking by exogenous ligands.

Discussion

The cell surface proteoglycan of NMuMG mouse mammary epithelial cells is a heparan sulfate–rich proteoglycan (Rapraeger et al., 1985) which, based on its lipophilic properties (Rapraeger and Bernfield, 1983, 1985), is believed to be embedded in the plasma membrane. Because of its ability to bind components in the extracellular matrix (Koda et al., 1985), the proteoglycan is thought to be a receptor that binds the cells to the matrix. This role implies that the proteoglycan should be localized on the basal surface of epithelial cells, i.e., in contact with the basement membrane, and should interact with the cytoskeleton. We now provide additional evidence for this role, demonstrating that (*a*) the cell surface proteoglycan becomes sequestered at the basolateral plasma membrane with the acquisition of cell polarity; (*b*) extraction with Triton X-100 distinguishes three fractions of cell surface proteoglycan: a fraction (~70%) presumed to be free in the membrane, a fraction (~10%) bound via a salt-labile linkage, and a nonextractable fraction (~20%); (*c*) the detergent-resistant fractions sequestered at the basal cell surface co-localize with intracellular F-actin; and that (*d*) cross-linking of the apical cell surface proteoglycan into clusters causes it to interact with the cytoskeleton.

These results indicate how the proteoglycan may act as a matrix receptor to stabilize the morphology of epithelial sheets. Residence of the proteoglycan at the basolateral cell surface is appropriate as it binds strongly to matrix components produced by the stromal cells that lie beneath the epithelium. Epithelial cells in sheets do not generally move in relationship to the matrix, thus explaining the anchorage of the proteoglycan to the cell not only by an association with the plasma membrane, but also by a linkage to the cytoskeleton, inferred from its resistance to detergent extraction and its close spatial relationship with actin at the basal cell surface. Indeed, the matrix may cross-link the proteoglycan at the cell surface, inducing its interaction, directly or indirectly, with the cytoskeleton, and thus stabilize the epithelial morphology.

Proteoglycan Becomes Sequestered on the Basolateral Cell Surface

Staining the apical surface of subconfluent NMuMG cells with a monoclonal antibody specific for the core protein of the cell surface proteoglycan demonstrates proteoglycan on almost all cells. However, after the cells reach confluence, an increasing number shows reduced amounts of apical proteoglycan. Ultimately, after 2 wk at confluence, the monolayers display little, if any, apical surface proteoglycan. This is corroborated by a reduction in the amount of proteoglycan released by treatment of the confluent monolayers with trypsin. Despite the disappearance of proteoglycan from their apical surfaces, confluent cells do express cell surface proteoglycan at their basolateral surfaces. This is demonstrated by antibody staining of basolateral surfaces exposed by EDTA or detergent treatment and is confirmed by stainings

of intact monolayers grown on nitrocellulose filters through which antibodies gain access to the basolateral cell surface. A similar dependence on cell density has been described for the distribution of surface antigens on MDCK cells (Ojakian and Herzlinger, 1984; Balcarova-Stander et al., 1984). Residence of the proteoglycan at the basolateral surface is consistent with a functional role at this site, potentially a role in cell-cell and/or cell-matrix binding (see below).

Although development of polarity must involve anchorage to the substratum, possibly involving the proteoglycan, binding to basal matrix components might not account for all of the proteoglycan being sequestered basolaterally, especially as NMuMG cells deposit scarce amounts of matrix during culture. Sequestration of proteoglycan on the basolateral surface might occur, however, via intracellular sorting mechanisms. Newly synthesized proteoglycan may be directed solely to the basolateral plasma membrane, but in subconfluent cells lacking established intercellular junctions the molecule may be free to diffuse in the membrane to the apical surface, a process prevented by the formation of complete junctions in older monolayers. Apical proteoglycan would be lost by shedding or intracellular degradation and not replaced. Alternatively, newly synthesized proteoglycan may be randomly inserted into the plasma membrane of subconfluent cells, resulting in an apical as well as basolateral distribution, but directed insertion into the basolateral membrane may occur after additional polarity is established.

Anchorage of Proteoglycan to the Cell

The lipophilic properties of the cell surface proteoglycan suggest that it is inserted by its core protein into the plasma membrane. This is supported (*a*) by the failure of heparin (Rapraeger and Bernfield, 1985), KCl, or enzymatic removal of the GAG chains to displace the proteoglycan; (*b*) by the extractability of a major fraction of the cell surface proteoglycan by nonionic detergents at neutral pH; and (*c*) by movement of the proteoglycan in the plane of the membrane. However, anchorage to the cell may involve more than a lipid interaction, as a fraction of the cell surface proteoglycan also resists detergent extraction and its proportion can be increased by cross-linking with exogenous ligands. Thus, the cell surface proteoglycan appears to be both embedded in the plasma membrane and bound to components in the detergent-insoluble residue.

Triton extraction at pH 5.0 fails to release cell surface proteoglycan despite the release of intracellular proteoglycan and GAG fragments. However, ~80% of the cell surface proteoglycan is released if the Triton mixture is supplemented with KCl, which apparently disrupts ionic interactions with components in or near the membrane that are stabilized at this pH.

Triton extraction at pH 7.5 distinguishes three fractions of the cell surface proteoglycan. Nearly 70% is released by Triton extraction at pH 7.5, which apparently fails to stabilize the ionic interactions seen at pH 5.0; this proteoglycan fraction is presumed to be free in the membrane. Approximately 10% is released by the Triton mixture supplemented with KCl, and ~20% is insoluble.

A similar pH-dependent extraction has been described for other membrane proteins, e.g., band 3 of erythrocytes (Yu et al., 1973), which is reportedly due to isoelectric aggregation of spectrin (pK_a of 4.8), bound to band 3 at the cytoplasmic face of the membrane (Nicolson, 1973; Golan and Veatch, 1980). Binding of actin to this complex is also stabilized in this pH range.

A recent report by Carey and Todd (1986) demonstrates that a fraction of cell surface heparan sulfate proteoglycan from Schwann cells resists extraction by Triton or salt alone, but is extracted by a combination detergent/salt treatment, as we report, from the cytoskeleton-rich residue, along with significant amounts of spectrin and actin.

In keeping with a potential cytoskeletal interaction, the distribution of F-actin at the basolateral surface of the NMuMG mammary epithelial cells is highly coincident with the cell surface proteoglycan fraction that resists detergent extraction at pH 7.5 (~33% of the total). This coincidence is most clearly seen at the basal cell surface where discrete actin filament bundles are observed and where the distribution of these bundles changes from irregularly-arranged filament bundles to highly organized, parallel fibers as the cells reach confluence and establish polarity. Cell surface proteoglycan localizes to each of these sites of F-actin organization and reflects the change in actin distribution as the epithelial sheets establish polarity. In a similar manner, Woods et al. (1984) reported that an antibody directed against the cell surface heparan sulfate proteoglycan of hepatocytes colocalizes with actin in stress fibers and at the margins of spreading fibroblasts. In addition, studies of fibroblast attachment to intact fibronectin or its fragments (Woods et al., 1986) demonstrate that the cell-binding domain itself is not sufficient to promote complete attachment and spreading; this domain together with either of the two heparin-binding domains is required for formation of stress fibers terminating in focal adhesions, such as seen with intact fibronectin, suggesting the involvement of a cell surface heparan sulfate proteoglycan in this cytoskeletal organization. In NMuMG cells, the simultaneous, coincident collapse of both the cell surface proteoglycan and the actin network in the presence of cytochalasin D further suggests an association between these materials. This association recalls the prior report (Rapraeger and Bernfield, 1982) that this cell surface proteoglycan is capable of binding to F-actin in vitro. Whether this binding has relevance to the physiological association suggested here remains to be established.

Significance of a Mobile Membrane-anchored Proteoglycan

The cell surface proteoglycan fractions that can be discerned based on their susceptibility to detergent extraction at different pH values or salt concentrations may represent different steps in the process of sequestration of proteoglycan at the basolateral cell surface, including initial insertion in the membrane, followed by anchorage to both the cytoskeleton and the extracellular matrix. For example, in fibroblasts, colocalization of fibronectin, heparan sulfate proteoglycan, and the cytoskeleton has been reported in substratum adhesion sites (e.g., Heggeness et al., 1978; Hynes and Destree, 1978; Woods et al., 1984). On epithelial cells, fibronectin, which binds heparan sulfate (Yamada et al., 1980; Stamatoglou and Keller, 1982) might serve to cross-link cell surface proteoglycans, causing the proteoglycan to become associated with and immobilized by the cytoskeleton. This in turn would promote further establishment of cell polarity.

Proteoglycan Is Assimilated into Clusters by Cross-

linking Ligands. Although it is unclear at present which, if any, basal extracellular materials are available in the NMuMG cell cultures to act as cross-linkers at the basal surface, exogenous antibodies apparently suffice to promote the association of apical cell surface proteoglycan with the cytoskeleton. Treatment of the cells with mAb 281 antibody alone does not affect the behavior of the proteoglycan. mAb 281, although bivalent, is likely to be an ineffective cross-linking ligand because, as it is a monoclonal antibody, it recognizes only one binding site on the antigen. In contrast, addition of a second, anti-rat antibody generates a multivalent immune complex capable of cross-linking adjacent proteoglycans. In this case, all of the apical surface antigen is found in distinct clusters. This occurs at 4°C or in the presence of azide, suggesting that the clustering is due to diffusion. Although the diffusion of proteins in membrane lipid is slowed at 4°C (Nicolson, 1976), antibody-induced cluster formation does occur (reviewed by de Petris, 1977). Staining with FITC-conjugated phalloidin demonstrates that actin is present in a punctate pattern before antibody staining, probably in the cores of microvilli, and that many of the proteoglycan clusters generated by antibody cross-linking correspond to these structures (not shown).

Cross-linking antibodies appear to cause the proteoglycan to associate with the cytoskeleton. Triton fails to extract proteoglycan clusters at neutral pH, suggesting that the proteoglycan becomes coupled to detergent-insoluble materials. When the proteoglycan is assimilated into clusters at 4°C, its diffusion becomes restricted and it does not form larger aggregates. Although the clusters do aggregate if the cells are warmed to 37°C, they fail to do so even after 4 h at 37°C if energy blockers or cytochalasin D are present, suggesting that they are tethered to an immobile component and are no longer free to diffuse and that aggregation of the clusters requires the actin microfilament network.

Other cell surface components are thought to be associated with the cytoskeleton when clustered. These include, for example, concanavalin A receptors on *Dictyostelium* (Condeelis, 1979), surface IgG on lymphocytes (Flanagan and Koch, 1978), and *N*-formyl chemotactic peptide receptors on the surface of human granulocytes (Jesaitis et al., 1984). Also, *Dictyostelium* plasma membrane containing receptors initially patched by concanavalin A bind to filamentous actin (Goodloe-Holland and Luna, 1984). Possibly, cytoskeleton-bound proteoglycan clusters form at sites of close cytoskeleton/membrane association, such as those in microvilli, proposed by Koch and Smith (1978) to be the site of the association between the actin cytoskeleton and surface H-2 antigen. However, it is also possible that the proteoglycan clusters are formed around other membrane proteins which, in turn, are anchored to the cytoskeleton, causing the proteoglycans to behave as if they were directly linked.

Anchorage of Matrix-bound Proteoglycan to the Cytoskeleton. Because of its anchorage to the cell, the cell surface proteoglycan could be a means by which cells orient matrix materials and bind the cells to the matrix, a process requiring both matrix recognition and action of the cytoskeleton. Heparan sulfate proteoglycans in general bind a variety of extracellular matrix components (Yamada et al., 1980; Stamatoglou and Keller, 1982; Sakashita et al., 1980; Lattera et al., 1983; Fransson et al., 1983) and the NMuMG cell surface proteoglycan binds specifically to interstitial collagens and fibronectin (Koda and Bernfield, 1984; Koda et al., 1985). Lipophilic heparan sulfate proteoglycans are present in substratum adhesion sites of fibroblasts cultured in vitro (Lark and Culp, 1984) and have been thought to be involved in cytoskeletal organization (Rapraeger and Bernfield, 1982; Woods et al., 1984; Woods et al., 1986; Carey and Todd, 1986). It remains to be shown, however, that the proteoglycan is a transmembrane protein.

The cell surface proteoglycan present on the basal surface of epithelia is a prime candidate to anchor cells to the underlying basement membrane. In addition, binding to components deposited by the underlying stromal tissue might provide a means of communication between these tissues. Binding a matrix component, thus cross-linking mobile proteoglycans, as done here with antibodies, and promoting an association of the proteoglycan with the cytoskeleton would stabilize epithelial morphology. The ultimate result of cross-linking by an insoluble extracellular matrix could differ from that by a soluble ligand such as an antibody. The insolubility of the matrix could restrict further aggregation, which leads to shedding or endocytosis with a soluble ligand, and a direct, stable adhesion between the matrix and the cytoskeleton would be established.

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